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Should we sync? Seascape-level genetic and ecological factors determine seagrass flowering patterns

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Running headline: Flower synchronisation in *P. oceanica*

Summary

1. Spatial and temporal heterogeneity in flowering occur in many plant species with abiotic pollination and may confer fitness advantages through mechanisms such as predator satiation or pollination efficiency. Environmental factors such as light quality or quantity and temperature play an important role in inducing synchronisation on wide geographic scales. On a smaller geographic scale, external factors such as resource availability and herbivory are theorised to trigger flowering, while genetic factors may also play an important role.
2. In this study, we assessed the importance of ecological and genetic factors in shaping seascape-level spatial heterogeneity in flowering of the seagrass *Posidonia oceanica*. By investigating spatially close sites (<20 km) with similar seascape configurations and depth, we assume that major environmental drivers (temperature and light) were equivalent.
3. We assessed four ecological factors (productivity, leaf nitrogen and carbon content and herbivory) and three genetic factors (heterozygosity, relatedness and clonality) to assess three hypotheses for synchronised flowering in *P. oceanica*: (1) clone synchronisation (internal clock hypothesis), (2) variation in nutrient availability, potentially caused by spatial heterogeneity in herbivory rates or nutrient translocation *via* clonal integration (resource budget hypothesis) or (3) kin selection and sibling synchronisation.
4. Internal relatedness and heterozygosity had a significant positive effect on the abundance of flowers. Moreover, productivity and genotypic richness (clonality) were negatively associated with flower density, although at a lower level of significance. In addition we found that clones were almost exclusively shared among mass-flowering patches and patches without mass-flowering, respectively.

5. *Synthesis*. The results shed new light on seagrass flowering patterns and on the mechanisms of flower synchronisation at the patch level within a wider spatial scale. We found support for the kin selection hypothesis and indirect evidence for the resource budget hypothesis. Thus a combination of mainly genetic but also ecological factors causes the observed heterogeneous flowering patterns in *Posidonia oceanica* seascapes. In addition, we found a strong positive relationship between the number of flowers and heterozygosity, adding evidence to the controversial association between heterozygosity and fitness when a limited number of loci are used. To our knowledge, this study is the first to link both ecological and genetic factors with flower abundance in a species with a presumed masting strategy.

Key-words: aquatic plant ecology, genetic diversity, herbivory, heterozygosity, internal clock, kin selection, relatedness, resource budget hypothesis, *Posidonia oceanica*, primary production

Introduction

For many flowering plants with abiotic pollination the likelihood of successful fertilisation depends upon the synchrony of sexual activity and the proximity of compatible mates (Knapp *et al.* 2001; van Tussenbroek *et al.* 2010). One strategy to address these limitations is mast seeding, which involves strong fluctuations of reproductive output by individual plants as well as synchronisation among individuals (Crone *et al.* 2009). This strategy, although not very common, has been described mainly in terrestrial plants, ranging from bamboo to *Dypterocarpacea* (Janzen 1974; 1976). Some marine plants also present similar synchronised reproductive fluctuations (e.g. Inglis & Smith 1998), as well as abiotic pollination, suggesting they may display a masting reproductive strategy. Mast seeding has important disadvantages, such as the decrease in frequency of reproduction or the likely higher density-dependent seedling mortality in mast years (Hett 1971; Waller 1979). However, evolutionarily, synchronisation of flowering and seed production may confer fitness advantages through mechanisms such as predator satiation or pollination efficiency to avoid pollen limitation (Kelly 1994, Kelly & Sork 2002). In some species, predators are satiated during mast years, with minor impact on adult individuals, while predator populations are kept in check during non-mast years. Moreover, pollination efficiency is high in mast years, but pollen becomes limiting in non-mast years. These observations explain why synchronisation may increase individual fitness, but do not explain the actual mechanisms of synchronisation (Crone *et al.* 2009). Determining the triggers of synchronisation can have important implications for understanding population dynamics and species distribution, as can the factors limiting reproductive effort. In fact, if those cues do not exist locally, populations in a given area may only subsist via asexual reproduction, with important implications for the future of that population (Honnay & Jacquemyn, 2008; Hughes & Stachowicz, 2009; Oliva *et al.* 2014; Jahnke *et al.* 2015a).

Masting species display several mechanisms of flower synchronisation that include internal and environmental cues (e.g. Taiz & Zeiger 2002). Light and temperature are recognised as the two main environmental signals that can provide a consistent prompt to initiate reproductive growth of plants. However, the exact mechanism of synchronisation may not be easily discernible and may be strictly related to environmental cues or to the plants' ability to gain resources (Crone *et al.* 2009 and references therein). Many plant species require more resources to flower and set seed than they gain in a year, and therefore flower only when they exceed some threshold amount of stored resources. In this context, clonal integration and the translocation of nutrients within physically connected clones could accelerate the acquisition of sufficient nutrients. Herbivory, as an external factor, could also blur these patterns by affecting resource acquisition (Planes *et al.* 2011). In contrast, in bamboo and other semelparous plants, the occurrence of synchronous flowering has been explained by an internal clock (Isagi *et al.* 2004). The duration of the internal clock is believed to be fixed for a given species, but the actual flowering year is dependent on the genotype (Isagi *et al.* 2004). On the other hand, synchronisation with neighbours might also be regulated via kin selection and the extent of flowering synchronisation might depend on the relatedness of the community (File *et al.* 2012) and the resulting balance between the overlap in niche use *vs.* cooperation between relatives. The different mechanisms and possible interactions can cause plants to have cyclical or chaotic patterns of reproduction over time (Isagi *et al.* 1997; Satake & Iwasa 2000). In summary, flower synchronisation may be mediated by external environmental cues related to resource availability or by internal cues related to clone synchronisation, genetic fitness or kin selection and sibling synchronisation.

In the marine environment, effective pollination presents a serious challenge, similar to terrestrial wind pollination systems. However, marine angiosperms (i.e. seagrasses) have evolved a number of traits suitable for a hydrophilous pollination strategy, such as filamentous pollen dispersed passively through water movement (Ackerman 2000). Perennial seagrasses are often characterised by high clonality, relatively low sexual reproductive output and large variation at different spatial scales in the distribution and abundance of flowers (Inglis and Smith, 1998; Arnaud-Haond *et al.* 2012). Indeed, asynchronous flowering at small spatial scales often results in a patchy distribution of flowers (Inglis & Smith 1998; van Tussenbroek *et al.* 2010), and might lead to low reproductive output due to geitonogamous or autogamous selfing. However, much of the pollen is likely to become entrained very locally because of synchronous leaf fluttering (Kendrick *et al.* 2012), and synchronisation with immediate neighbours might, at the centimetre scale, represent the only strategy to ensure pollination – particularly in monoecious seagrass species. Furthermore, given that also 'long-distance' subaqueous pollen transport is limited mostly to the range of metres (Zipperle *et al.* 2011; McMahon *et al.*, 2014; Sinclair *et al.* 2014) synchronisation at small to medium spatial scales (i.e. patch, cove) may be crucial to prevent pollen limitation, while ensuring outcrossing where the maximum dispersal distance exceeds clonal range (Sinclair *et al.* 2014).

Posidonia oceanica (L.) Delile is a long-lived and slow growing Mediterranean endemic seagrass. It is an ecosystem engineer and forms monospecific meadows that provide important ecosystem services, such as sediment stabilization and acting as a nursery for juveniles of multiple commercially-important species (Diaz-Almela & Duarte 2008). *Posidonia oceanica* is a monoecious species that can reproduce asexually by lateral elongation of rhizomes, and sexually with hermaphrodite flowers (Ackerman, 2006). Flowers

appear between September and November (Buia & Mazzella 1991; Calvo *et al.* 2010), the hydrophilic pollen being released into the water column and surviving for several hours during which it is dispersed by local currents (Kendrick *et al.* 2012). Seeds ripen five months after the initiation of flowering (Buia & Mazzella 1991) and float to the surface, where they can be transported for one to three weeks by surface currents and wind-forcing until they sink and germinate (Serra *et al.* 2010). Flowering patterns in this plant exhibit important spatio-temporal variations: there are high-prevalence years, when 80% of meadows over large geographical areas flower, and other years when only 3% of meadows flower (Diaz-Almela *et al.* 2006). At a smaller spatial scale, even in flowering years the distribution of flowers within meadows is often patchy (Diaz-Almela *et al.* 2006). The episodic synchronisation of flower and fruit production in *P. oceanica* can be considered a masting strategy. Masting in *P. oceanica* may be advantageous and increase individual fitness for two main reasons: first, herbivorous fish are known to preferentially feed on flowers (Vergés *et al.* 2007), thus, predator satiation may be necessary to ensure a high proportion of successful seeds and to lower the impact on the adult plants; and second, in the marine realm, where pollinators are absent and pollen dispersal is limited, synchronisation of flowering with neighbouring plants might be crucial for successful fertilization. However, there is a lack of studies addressing the proximate mechanisms mediating flowering in *P. oceanica* and assessing whether masting is favoured in this species.

In 2011, we observed a flowering event in several naturally fragmented *P. oceanica* meadows along the Catalan coast, in the NW Mediterranean. While the investigated patches were at a similar depth only tens of metres apart and thus were exposed to corresponding environmental cues (i.e. temperature and light availability; Inglis and Smith, 1998; Diaz-Almela *et al.* 2006; Montefalcone *et al.* 2013), they presented contrasting flower abundances.

The main hypotheses considered to explain the observed patterns and the potential mechanisms of flower synchronisation within and among patches included: (1) clone identity and clone synchronisation (internal clock), (2) variation in nutrient availability per individual patch, potentially caused by spatial heterogeneity in herbivory rates or nutrient re-location *via* clonal integration (resource budget) or (3) kin selection and sibling synchronisation. Moreover, we also investigated if levels of genetic diversity, specifically heterozygosity as a proxy for individual fitness, differed between patches with high or low flower abundance.

Material and Methods

Environmental variables

In October 2011, we noticed a flowering event in several *P. oceanica* seagrass meadows along the Catalan coast. We selected three shallow (5-8 m depth), naturally fragmented meadows with a similar seascape configuration that were several kilometres apart (<20 km) (Fig. 1) and that had been assessed for levels of herbivory and internal resources in another study three months previously (Pagès *et al.* 2014). For each of these three meadows, we identified three patches with mass-flowering and three patches without mass-flowering. Sampling in mass-flowering patches and patches with a low/no density of flowers enabled us to investigate potential drivers of flowering synchronicity, measured as density of flowers per patch. Patches were generally small (mean size 5.6 ± 0.7 m^2) and were all on sandy substrate. We measured flower abundance and shoot densities in 40 x 40 cm quadrats (4 replicates per patch; 6 patches - 3 with mass-flowering and 3 without mass-flowering - per site, total n = 24 per site) to control for possible variability in flower or shoot densities among sites. We also sampled five flowering and five non-flowering shoots in mass-flowering patches, and 10 non-flowering shoots in patches without mass-flowering for genetic analyses (see below). Additionally, in each of the patches we collected shoots with long rhizomes (ca. 15 cm; with

189 and without flowers for the mass-flowering patches, and without flowers for patches without
190 mass-flowering) in order to reconstruct the frequency of inflorescences for the past seven
191 years at the level of the shoot/patch/site using a lepidochronological approach (e.g. Pergent &
192 Pergent-Martini1990, Balestri & Vallerini 2003) (total n = 114 shoots analysed).
193
194 Three months before the flowering event, in July 2011, we assessed leaf nitrogen and
195 carbon content, direct herbivory rates and leaf growth (as a surrogate of primary production)
196 on the same three sites and seagrass patches with and without mass-flowering (3 + 3 = 6
197 patches per site) for another study (Pagès *et al.* 2014). Nitrogen and carbon were measured
198 for each of five randomly chosen shoots per patch (pooled), for which the leaves were
199 cleaned of epiphytes, dried until constant weight and ground. The samples were then sent to
200 the Unidade de Técnicas Instrumentais de Análise (Universidade de Coruña) where nitrogen
201 and carbon concentrations were measured using an elemental analyser EA1108 (Carlo Erba
202 Instruments). Primary production was estimated using a modified Zieman's method (Zieman
203 1974; Pérez & Romero 1994), and herbivory was assessed with a tethering technique similar
204 to that of Prado *et al.* (2007). SCUBA divers marked five shoots per patch (5 × 6 = 30 shoots
205 per site). For each shoot, we marked the leaves' base by piercing them with a needle to
206 measure leaf elongation. We also recorded the initial number of leaves, the initial leaf length
207 and the state of the apical part of each leaf (broken, eaten by fish, eaten by sea urchin or
208 intact). Fifteen days later, all marked shoots were collected and transported to the laboratory
209 for processing. We counted the number of leaves on each shoot and measured the length and
210 state of the apex of each leaf on the shoot. For each leaf, the new leaf tissue produced
211 (between the pierced mark and the ligula) was also measured (i.e. leaf elongation). Primary
212 production (cm $shoot^{-1}$ day^{-1}) of pierced shoots was determined by dividing the length of new
213 tissue produced by the number of days elapsed since marking. Shoot herbivory rates (cm

214 shoot^{-1} day^{-1}) were estimated for each of the collected shoots by adding leaf elongation (cm
215 of new tissues produced) to the initial length and subtracting this total from the final leaf
216 length, finally divided by the number of days elapsed since marking (Prado *et al.* 2007). Only
217 leaves that had clear herbivore bite marks were assigned to herbivory and the rest were
218 discarded to avoid herbivory overestimation.
219
220 *Sampling of genetic material, DNA extraction and microsatellite analyses*
221 In order to test whether the very contrasting abundances of flowers among patches
222 within each site could be related to the distribution of genotypes or the distribution of
223 genotypic richness and genetic diversity among patches, we analysed the shoots collected in
224 mass-flowering patches (n = 5 flowering + 5 non-flowering = 10 shoots) and in patches
225 without mass-flowering (10 non-flowering shoots) (see above). This resulted in a total of 176
226 individual samples (60 shoots per site, 3 sites, 4 shoots were discarded), which were cleaned
227 of epiphytes, dried and stored in silica crystals. For DNA extraction, an approximately 6 cm
228 long, dry *P. oceanica* leaf fragment was homogenized with a TissueLyser MixerMill
229 (Qiagen) for 3 min at a frequency of 12 oscillations/second. DNA was extracted from the
230 pulverized samples with the NucleoSpin® 96 Plant II kit (Macherey-Nagel), following the
231 procedure described in Tomasello *et al.* 2009.
232
233 Twenty-eight polymorphic microsatellites were used for the analysis, which included
234 twelve putatively neutral microsatellites (Procaccini & Waycott 1998; Alberto *et al.* 2003), as
235 well as 16 EST-linked microsatellites (Arranz *et al.* 2013). The previously commonly used
236 locus Po 5-49 was not used in this analysis and the reverse primer of Po 5-40 (Alberto *et al.*
237 2003) was replaced with a new primer (Po 5-40M: 5'-
238 CATGTTATAATCCTTTGTATGGAGGT-3'). Microsatellites were combined in four

239 different multiplexes and all PCRs were run under the following conditions: 95ºC for 15 min,
240 35x (94ºC for 30 sec, 60ºC for 1min 30 sec, 72ºC for 1 min), with a final annealing step of
241 60ºC for 30 min. Scoring was performed following Migliaccio *et al.* (2005) and Tomasello *et*
242 *al.* (2009).
243
244 *Genetic data analyses: clonal identification, genetic diversity and relatedness*
245 Clonal discrimination and identification of multilocus genotypes (MLGs) and
246 multilocus lineages (MLLs) were performed using the software GenClone (Arnaud-Haond &
247 Belkhir 2007) and through the calculation of P_{sex}, the probability that identical MLGs derived
248 by chance from sexual reproduction versus those that are actual clones. After MLG
249 identification, clones were removed so that only unique MLGs were present in each category.
250 However, as somatic mutations and scoring errors could lead to an underestimation of the
251 number of clones, the data set of MLGs was further investigated by removing one locus at a
252 time to identify MLGs that are distinct at one locus, termed MLLs, and re-calculating P_{sex}.
253 Calculated P_{sex} probabilities were all lower than 0.01, which is the level that was used to
254 reject the null hypothesis that the ramets belong to individuals derived from distinct sexual
255 events (Serra *et al.* 2007). We used MLGs for all following statistics, but the number of
256 MLLs is also reported. We also pooled samples from all patches within locations to identify
257 MLGs that might be shared among patches. Genotypic richness (clonality) was estimated
258 according to Dorken & Eckert (2001): $R = (G-1)/(N-1)$, with G representing the number of
259 genotypes and N representing the number of sampled shoots. Genomic diversity
260 measurements were calculated using GenAlex 6.5 (Peakall & Smouse 2012). The fixation
261 index and significance were calculated using GENETIX 4.05 (Belkhir *et al.* 2001) with 1000
262 bootstrap replicates. Individual heterozygosity was calculated using genhet (Coulon 2010),
263 which calculates the proportion of heterozygeous loci in an individual (PHt) and the

standardized expected and observed heterozygosities (Hs_exp and Hs_obs) based on PHt. Finally, to assess whether kinship could regulate flowering synchronisation, internal relatedness within patches or categories was calculated using the software Storm (Frasier 2008).

Statistical analyses

We used generalised linear models (GLM) to investigate the patterns and mechanisms of synchronicity in flowering among seagrass patches. To do that, we tested the effects on the response variable 'flower density per patch' (abundance of flowers per square metre per patch, n = 6 patches per site, 3 sites) of the fixed continuous variables herbivory, percent nitrogen content, percent carbon content and primary production, as environmental resource-related variables to assess the resource-budget hypothesis; genotypic richness to assess the internal clock hypothesis; shoot relatedness within patches to evaluate the kin/sibling selection hypothesis; and individual heterozygosity as a surrogate for fitness and a potential factor further influencing the synchronicity of flowering. All statistical analyses were run considering the mean of each variable per patch as different shoots were considered for each of the measured variables (i.e. patch was considered the experimental unit, n = 6 per site). We considered the possibility of including the random effect 'site' into the model to account for the variance among measurements taken from the same site (three levels, the three sites), but Akaike Information Criterion (AIC) did not support the inclusion of this random effect. The final model was thus a GLM with a negative binomial distribution (theta = 1.143) to account for the existence of extreme counts in the response variable 'flower density'. We started model selection with a full model including all explanatory variables. Then, each fixed effect was dropped one by one in a stepwise backward selection procedure using the Akaike Information Criterion (AIC) and the likelihood ratio test statistic (Zuur *et al.* 2009). We also

conducted a stepwise forward selection procedure that lead to the same best-selected model, adding robustness to the chosen model (see supplementary PosiFlower_Rmarkdown.html file). We also tested the inclusion of some interactions into the best-selected model variable due to the impossibility of the model to converge with more complex designs (only double interactions were tested, see supplementary PosiFlower_Rmarkdown.html file). Normality and homogeneity of variances were checked graphically by inspecting residuals and fitted values. The residuals of the response variable 'flower density' followed the assumption of normality after fitting the model. Even though we used a negative binomial distribution, the final model still displayed a small degree of overdispersion ($\Phi = 1.34$), which should be considered when interpreting marginally significant fixed effects. Data were analysed with the package lme4 and MASS in the statistical software R (Venables & Ripley 2002; R Development Core Team 2012; Bates *et al.* 2014) (see complete model selection procedure in the supplementary file PosiFlower_Rmarkdown.html). We used the package visreg to visualise the effects of each predictor on the response variable with the fit from the multivariate best-selected model and to visualize the combined effects of 2 predictors to the response variables (Breheny & Burchett 2014).

We used a linear model to analyse the effects of the fixed continuous variables herbivory, percent nitrogen content, percent carbon content primary production, heterozygosity and genotypic richness (clonality) on the dependent variable 'relatedness'. Model selection was performed following the same protocol as above (using AIC). The residuals of dependent variable 'relatedness' fulfilled the assumptions of normality and homoscedasticity after model fitting. We also used a linear model to analyse whether shoot density was different between mass-flowering patches and patches without mass-flowering

313 (factor ‘patch status’, fixed with 2 levels). Normality and homoscedasticity assumptions were
314 again fulfilled.
315

316 # Results

317 *Number of flowers in mass-flowering patches and patches without mass-flowering*

318 The average number of flowers was 97 ± 26 flowers m^{-2} in mass-flowering patches
319 and 5 ± 2 flowers m^{-2} in patches without mass-flowering. Differences in flower abundance
320 were not linked to contrasting shoot densities between patches (P = 0.3) (mean shoot density
321 154 ± 8 shoots m^{-2}). The lepidochronological analysis of shoots yielded no signals of
322 flowering events in any shoot for the seven years before 2011, confirming the rarity of sexual
323 reproduction events in the assessed meadows. This was true both for non-flowering and
324 flowering shoots.
325

326 *Population genetic analyses: Heterozygosity, relatedness and genotypic richness*

327 We used a high number of microsatellites on a small spatial scale and 10 out of the 28
328 microsatellites proved uninformative in this analysis. Despite the small geographic scale,
329 genotypic richness (i.e. the number of clones) (R_{MLG}) – calculated by pooling mass-flowering
330 patches and patches without mass-flowering for each site – was high, ranging from 0.52 to
331 0.78 (Table 1, see Table S1 for single-patch values). We did not observe a clear difference in
332 genotypic richness between patches with and without mass-flowering. Flowering shoots
333 belonged to many different genotypes. Up to three genotypes (MLGs) were shared among the
334 different patches within each site, but almost without exception, the shared genotypes among
335 mass-flowering patches were different to those shared among patches without mass-flowering
336 (Table S2, Fig. 1). The exception occurred at Cabdells (Fig. 1b) where one MLG was shared
337 between a mass-flowering patch and a patch without mass-flowering. All patches (with and

without mass-flowering, respectively) at Giverola and Fenals shared at least one clone (Fig. 1a,c). The highest number of shared clones (n = 3) occurred between two patches without mass-flowering at Fenals (Fig. 1c). Between-sites clone sharing occurred only between Giverola and Cabdells. Two different MLGs were found in a mass-flowering patch at Giverola (both were not flowering) and a patch without mass-flowering at Cabdells. Another MLG was found with three representatives in a mass-flowering patch in Cabdells and one representative each at two patches without mass-flowering at Giverola.

Allelic richness and heterozygosity were similar in patches with and without mass-flowering at each location, ranging from 1.61 to 2.11 (allelic richness standardized to 16 genotypes) and from 0.347 to 0.402 (observed heterozygosity) (Table 1, see Table S1 for single-patch values). The fixation index F_{is} was negative and differed significantly from expectations under the Hardy-Weinberg equilibrium at all locations, indicating an excess of heterozygosity (Table 1). Average individual heterozygosity was generally higher in the mass-flowering patches (Table S3), and it was not higher in frequently found genotypes compared to genotypes that were only found once (Table S3).

Not all shoots belonging to the same genotype within the same patch flowered at the same time. Conversely, some shoots with identical genotypes did flower at the same time even if they grew in separate patches, where consequently clonal integration or direct communication was not possible (Table S2). Genotype relatedness within patches differed widely, ranging from -0.499 to 0.841 (Table S1).

Combined factors to predict flower synchronisation

GLM results indicated that both genetic and environmental factors influenced flower density per patch. However, genetic factors appeared to dominate over environmental ones in determining flower density per patch at the assessed scale. There was a significant positive relationship between flower abundance per patch and genetic relatedness as well as between flower abundance and individual heterozygosity (Table 2, Table S4, Fig. 2a,b). These results imply that higher relatedness and heterozygosity within a patch result in higher abundance of flowers (or alternatively that historically high flowering rates in these patches resulted in high heterozygosity and high relatedness). In fact, the best-selected model predicted an additive effect of heterozygosity and relatedness on flower densities per patch, with the highest density of flowers in patches with both high relatedness and high heterozygosity (Fig. 3). The combined effects of the rest of pairs of selected predictors on flower density per patch can be found in the supplementary (Fig. S1). Moreover, we also found that the abundance of flowers per patch was negatively related to vegetative tissue production (Table 2, Table S4, Fig. 2c) and genotypic richness (clonality) (Table 2, Table S4, Fig. 3d), implying that the higher the production of vegetative tissue, and the more clones/sample in a patch, the lower the abundance of flowering shoots (see Fig. S1). However, care should be taken when interpreting these last two results as the statistical significance was marginal (Table 2) and the model shows some degree of overdispersion (see Materials and Methods). The effect of each selected predictor to the response variable flower density can be inspected in the logarithmic link scale (the one used to fit the GLM) in the supplementary (Fig. S2). The variables herbivory, percentage of nitrogen and carbon in leaves, and all interactions had no effects on flower abundance per patch and were thus dropped from the model (see supplementary PosiFlower_Rmarkdown.html file). We could not test the effects of higher order interactions due to computational restrictions (model convergence impossible). The best-selected model, i.e. the model including the fixed effects heterozygosity, internal relatedness, genotypic

richness and production, explained 63.7% of total deviance (Table 2). Further, we found a positive relationship between relatedness and leaf nitrogen content ($P = 0.04$), suggesting that shoots with similar genotypes had higher leaf nitrogen contents.

Discussion

Flowering events are rare in *P. oceanica* meadows. The 2011 event was clearly unusual, with no prior flowering detected with reconstructive techniques (lepidochronology) in these meadows in the previous seven years. The spatial heterogeneity in this mast flowering event gave us a unique opportunity to identify mechanisms of flower synchronisation between patches with mass-flowering and patches without mass-flowering. Our results indicate that genetic factors played a major role in driving flowering synchronicity within and between mass-flowering patches: both relatedness among genotypes and heterozygosity were clearly associated with flower abundance. The former indicates that kin selection is a potential mechanism of spatial synchronisation, while the latter indicates increased fitness of mass-flowering patches. The negative correlation of vegetative tissue production with flower abundance per patch suggests that patch-level resource availability may also be a factor in mediating mast strategies in *P. oceanica*. Moreover, genotypic richness correlated negatively with flower abundance suggesting that – taken together with the findings on resource availability – clonal integration might also play a role. The strategy of kin selection as a mechanism of synchronisation, together with the observed increase in heterozygosity indicate that fitness, cooperation and decreased competition between closely related individuals may account for an increased ability to invest in sexual reproduction.

A mechanism frequently advocated to explain how flowering in different individual plants – mainly semelparous species – may get entrained, is the assumption of an '**internal**

412 **clock**', which would synchronise flowering in identical or closely related genotypes (John &
413 Nadgauda 1999). However, in our study we did not find evidence for such an 'internal clock'.
414 Indeed, we found that a high number of different genotypes flowered in different patches at
415 the same time and that not all shoots of identical genotypes flowered simultaneously.
416 Nevertheless, although different genotypes flowered together, clone identity still played an
417 important role: we found that identical genotypes were only shared within/among mass-
418 flowering patches or within/among patches without mass-flowering respectively, but not
419 between each group of patches (except for one case out of 18, where an identical MLG
420 occurred both in patches with and without mass-flowering, see Fig. 1b).
421
422 Many plant species require a minimum amount of resources to flower and set seed,
423 and therefore flower only above some threshold of stored resources (Crone & Rapp 2014).
424 The '**resource budget hypothesis**' has been observed to be the main mechanism of
425 synchronisation in some abiotically pollinated perennial grasses (Crone *et al.* 2009; Crone &
426 Rapp 2014). Indeed, we found a negative relationship between vegetative tissue production in
427 summer and flower abundance in autumn (albeit at a low level of significance). These results
428 highlight the inherent trade-off associated with allocating resources to reproductive or
429 vegetative organs. Previous studies with the same species have suggested that flowering has a
430 negative correlation with leaf biometry, rhizome elongation and production (Gobert *et al.*
431 2001; Gobert *et al.* 2005; Calvo *et al.* 2010) and that recovery from the stress induced by
432 sexual reproduction may take two years (Calvo *et al.* 2006). This evidence for a noticeable
433 impact of flowering events on shoot performance adds to the argument that flowering in *P.*
434 *oceanica* is expensive in terms of resources. As such, it is plausible that the negative
435 association between flowering and vegetative production in the preceding summer reflects
436 the conservation of resources to sustain the subsequent resource-intensive flowering. In

437 contrast, leaf nitrogen and leaf carbon content collected three months before the mast
438 flowering did not have a significant effect on flower abundance. Despite the fact that
439 herbivory may further affect individual nutrient levels, and has been shown to negatively
440 affect *P. oceanica* flower abundance in highly grazed meadows (Piazzi *et al.* 2000; Planes *et*
441 *al.* 2011), we did not detect significant effects of herbivory on the abundance of flowers in
442 this study either. Clonal integration and resource translocation between physically connected
443 clones may further complicate the resource budget of an individual plant within a patch
444 (Prado *et al.* 2008), but we did not directly assess this complex process in the present study.
445 The lack of correlation between flower abundance, herbivory, nitrogen and carbon content
446 could be partly due to a mismatch between our sampling time of these variables (three
447 months before flowering) and flower induction (up to seven months before flowering)
448 (Gobert *et al.* 2001). Moreover, we only measured nitrogen and carbon content of leaves;
449 while in seagrasses most carbon storage takes place in the rhizome (Alcoverro *et al.* 2001,
450 Roca *et al*. 2014). Finally, whereas the genetic make-up is a permanent characteristic of a
451 plant, and patch genetic structure may require years to decades to change (see for instance
452 Zupo *et al.* 2006; Jahnke *et al.* 2015a), nutrient levels and the amount of herbivory may have
453 changed between the time of flower induction and when ecological data were collected.
454
455 **Kin selection and sibling synchronisation** could also help to explain flower
456 synchronisation in *P. oceanica*, although this is a process that has been much less studied. We
457 assessed internal relatedness based on the occurrence and frequency of alleles at all 28 loci
458 and found a significant positive relationship with the abundance of flowers per patch. Thus,
459 the higher the relatedness of unique genotypes in a patch, the higher the abundance of flowers
460 in that patch. A recent study in *Z. marina* found that increased relatedness of experimental
461 and natural meadows resulted in higher shoot densities (Stachowicz *et al.* 2013). In the

462 absence of inbreeding, the expected value for unrelated individuals is 0, while parent-
463 offspring or fullsib relatedness values have an expected value of 0.5 (Queller & Goodnight
464 1989). Relatedness values in our study can be as high as 0.841 and are comparable to those
465 from a study with *Z. marina* also at a seascape level (Kamel *et al.* 2012). The generally very
466 high relatedness values in both seagrass species (often higher than expected from parent-
467 offspring relationships) can be explained by potential inbreeding (parent-offspring) and by
468 the fact that reproduction is predominantly asexual, with possibly common somatic
469 mutations, which may also be transferred to offspring in plants. Kin selection might increase
470 the competitive ability of more related patches when considering the trait sexual
471 reproduction.
472
473 The observed heterogeneity in flower densities is also associated with spatial
474 heterogeneity in heterozygosity, which has been linked to components of fitness in numerous
475 studies across a wide range of taxa (Di Fonzo *et al.* 2011). Specifically, high mean patch
476 individual heterozygosity was associated with high flower abundances per patch (see Fig.
477 2b). Although it is widely accepted that genome-wide heterozygosity is linked with overall
478 fitness, the debate remains whether a low number of molecular markers is able to reflect
479 genome-wide heterozygosity (reviewed in Hansson & Westerberg 2002). Our study supports
480 the link between heterozygosity and fitness (when fitness is defined as sexual reproductive
481 output, *sensu* Darwin 1872) even using a limited number of loci (28), since we found a strong
482 positive relationship between the number of flowers and heterozygosity (see Fig. 2b). In
483 contrast to several studies in seagrasses that associated high heterozygosity with big clones in
484 so called "general-purpose-genotypes" (Lynch 1984), in this study heterozygosity did not
485 differ significantly between genotypes that were only observed once and common genotypes
486 (Table S3).

All in all, our results shed new light on seagrass flowering patterns and on the mechanisms of flower synchronisation at the patch level within a wider seascape. We found support for the kin selection hypothesis and indirect evidence for the resource budget hypothesis. Our results support that an interaction between genetic factors (relatedness, heterozygosity and genotypic richness) and ecological factors (leaf production) cause the observed heterogeneous flowering patterns in *P. oceanica* seascapes. In addition, we found a strong positive relationship between the number of flowers and heterozygosity, adding evidence to the controversial association between heterozygosity and fitness when a limited number of loci are used. While there is a body of literature associating heterozygosity with fitness, research on implications of neighbourhood and kinship in seagrasses has only recently been initiated (Kamel *et al.* 2012; Stachowicz *et al.* 2013) and still deserves further research. Results presented here and results for the seagrass *Z. marina* (Stachowicz *et al.* 2013) indicate that cooperation and decreased competition between closely related individuals may account for fitness advantages, apparent in either higher levels of sexual reproduction (our study) or increased biomass accumulation (Stachowicz *et al.* 2013). Considering only our results, the opposite explanation is, however, also possible. A shoot growing among closely related individuals might be exposed to increased competition, because of higher niche overlap (Rautiainen *et al.* 2004). In evolutionary terms, it may therefore be more beneficial for an individual to invest in sexual reproduction, instead of asexual propagation. While asexually produced plants will encounter high levels of kin competition, sexually produced seeds, in contrast, may disperse further aided by currents and may establish in meadows where their genotype and phenotype are dissimilar to the neighbouring plants, decreasing niche overlap. Indeed, kin competition has been shown to play a role in determining flowering intervals in bamboo (Tachiki *et al.* 2015). Both scenarios

(kin cooperation and sibling competition) assume that kin recognition is possible in seagrasses. Although, to our knowledge, it has not been investigated for any seagrass species, results from terrestrial plants indicate that kin recognition is most likely mediated via root exudates (Biedrzycki *et al.* 2010), a form of intra-specific communication that should equally be possible in the marine environment. Another study on a terrestrial plant moreover confirmed that soil leachates might play an important role in flowering synchronization among neighbours (Falik *et al.* 2014).

To our knowledge, this study is the first to link both ecological and genetic factors with flower abundance in a species with a presumed masting strategy. These findings help to understand seascape-level synchronisation of individual but spatially close plants during mast flowering events and open new doors for exploring the role of relatedness in ecosystem functioning.

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Data accessibility

Data on herbivory, carbon and nitrogen leaf content and production on the patch level are deposited in Dryad repository (doi:10.5061/dryad.sj6dv; Jahnke *et al.* 2015b). An Rmarkdown html file with the R scripts used for model selection is available online as supporting information (PosiFlower_Rmarkdown.html).

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1. Estimate of population genetic parameters per patch of *Posidonia oceanica*.

Table S2. Number and frequency of sampled genotypes of *Posidonia oceanica*.

Table S3. Average proportion of heterozygous loci in an individual (PHt) and standardized observed and expected individual heterozygosity measurements (Hs obs and Hs exp).

Table S4. Coefficient estimation obtained by fitting a generalised linear model with the variables heterozygosity, relatedness, genotypic richness and production.

Figure S1. Combined effects of selected pairs of predictors on flower density per patch.

Figure S2. Effects of each of the selected predictors to the response variable flower density per patch in the logarithmic link scale.

Tables

Table 1. Estimate of population genetics parameters for *Posidonia oceanica* patches with mass-flowering (F) and without mass-flowering (NF) at each location. Allelic richness, heterozygosity and F_{is} measurements are based on the number of MLGs and therefore not standardized (with the exception of A_{16}). Ten out of 28 loci were uninformative (monomorphic). The following estimators are reported: N = number of individuals, %Pol = percent of polymorphic loci, MLG = multilocus genotype, MLL = multilocus lineage, R_{MLG} =(MLG-1)/(N-1), R_{MLL} =(MLL-1)/(N-1), Na = No. of Alleles/Locus, A_{16} = standardized allelic richness for the lowest number of samples, Ho = Observed Heterozygosity, He = Expected Heterozygosity, F_{is} = Fixation Index, * indicates significant F_{is} values. Site legend: GIV = Giverola, CAB = Cabdels, FEN = Fenals

Remark: FEN NF has an unequal N, as one locus did not amplify after three trials and its absence was therefore considered informative

Name	N	%Pol	MLG	MLL	R_{MLG}	R_{MLL}	Na (SE)	A_{16}	Ho (SE)	He (SE)	F_{is}
GIV NF	30	64	21	16	0.69	0.52	2.071 (0.230)	2.02	0.396 (0.075)	0.276 (0.047)	-0.414*
GIV F	30	57	21	11	0.69	0.35	1.929 (0.218)	1.91	0.354 (0.077	0.249 (0.049)	-0.401*
CAB NF	28	68	22	17	0.78	0.59	2.107 (0.214)	2.01	0.347 (0.074)	0.270 (0.046)	-0.266*
CAB F	30	68	16	12	0.52	0.38	2.107 (0.208)	2.11	0.395 (0.073)	0.311 (0.050)	-0.241*
FEN NF	29	61	17.9	13	0.59	0.41	1.893 (0.173)	1.88	0.385 (0.077)	0.285 (0.050)	-0.323*
FEN F	28	54	16	11	0.56	0.37	1.607 (0.119)	1.61	0.402 (0.087)	0.237 (0.045)	-0.681*

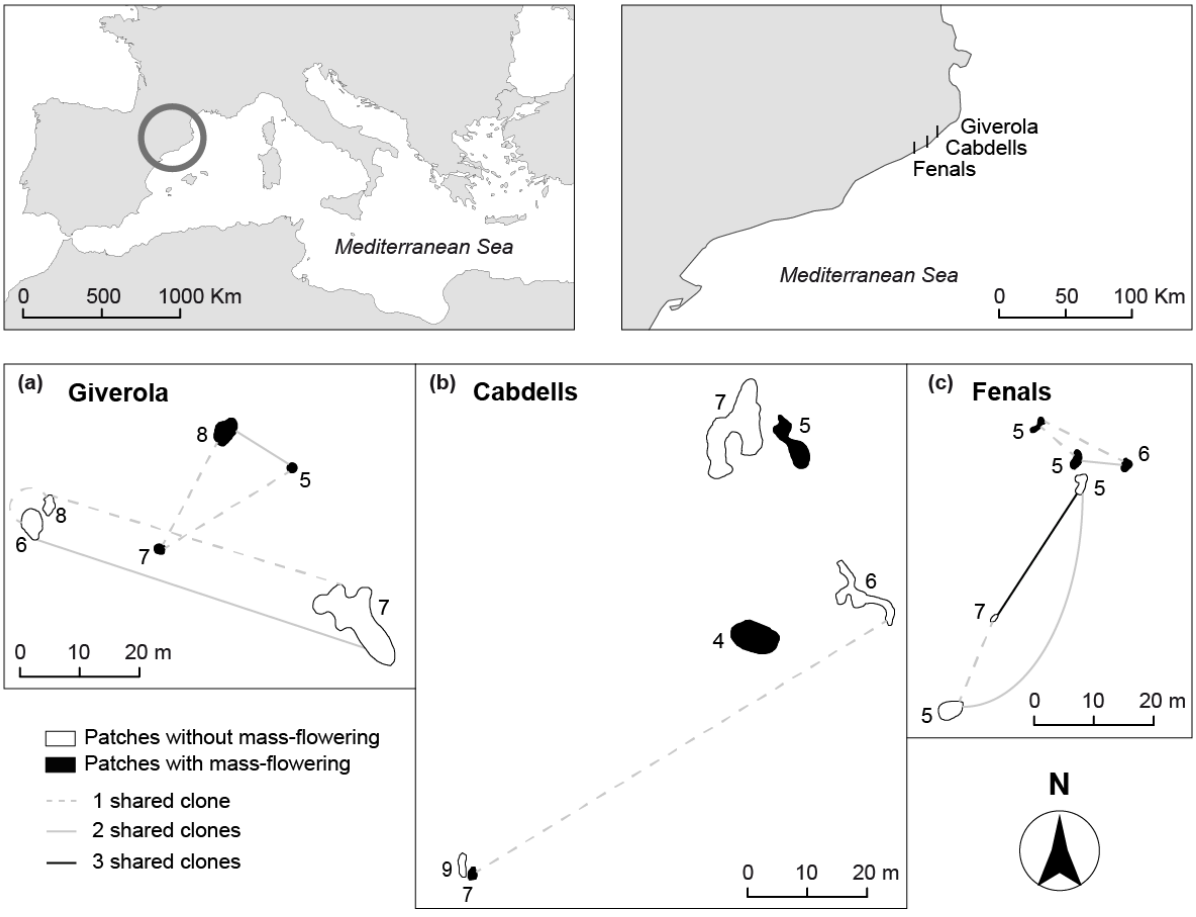
Table 2. Significance of predictors of the best-selected generalised linear model on the dependent variable 'Density of flowers per patch'. Change in deviance and corresponding Chi-square *P*-values for each predictor variable are computed by sequentially dropping them one by one. Model coefficients for each of these variables can be found in Table S4.

Effect	**Df**	**Dev**	**Residual Df**	**Residual Dev**	***P***
Null			15	40.67	
Heterozygosity	1	9.60	14	31.08	0.00**
Relatedness	1	9.50	13	21.58	0.00**
Genotypic richness	1	3.04	12	18.54	0.08·
Production	1	3.79	11	14.75	0.05·

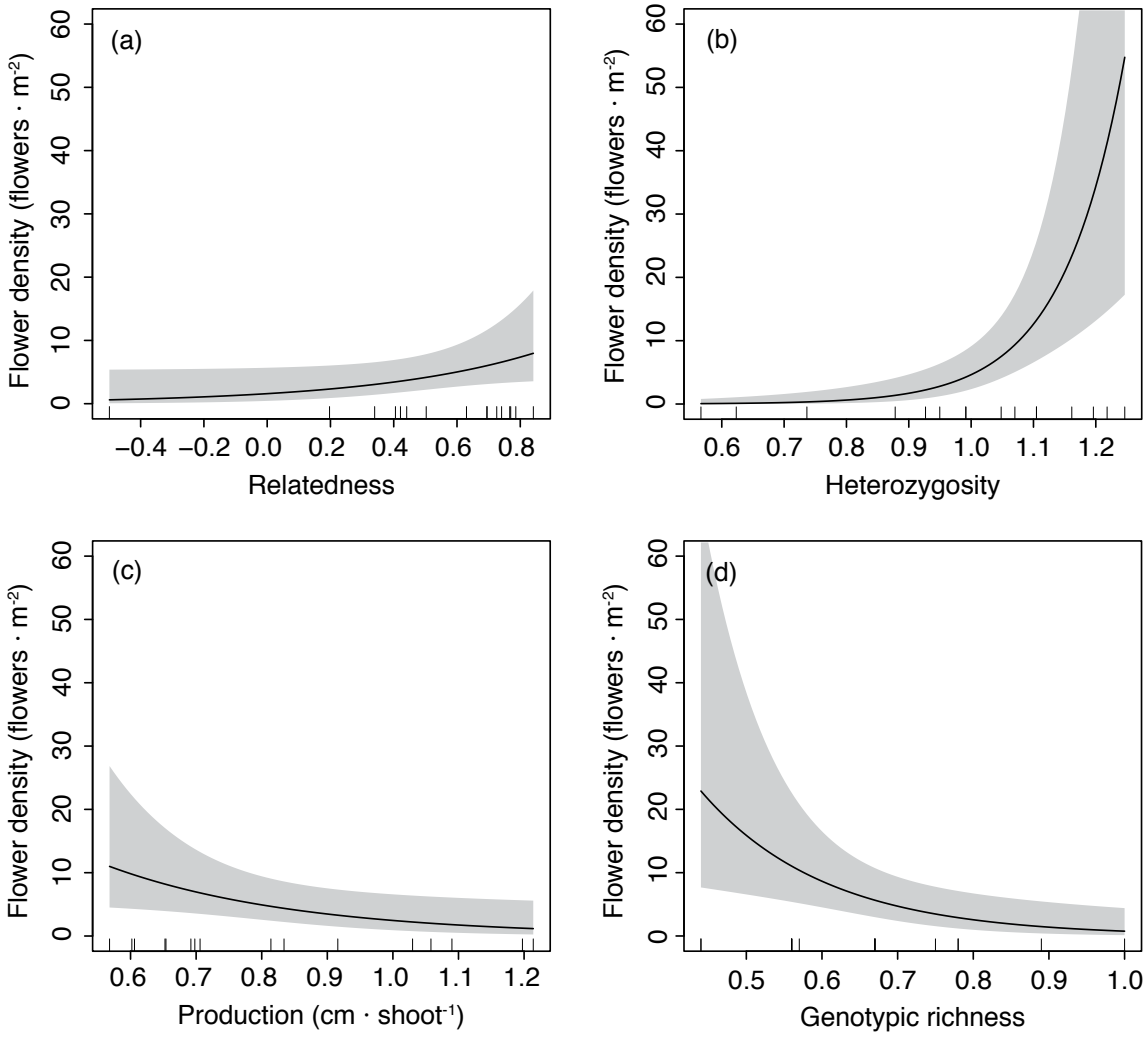
Significance codes: <0.001 '***' <0.01 '**' <0.05 '*' <0.1 '·' Df: Degrees of freedom. Dev: Deviance.

Figures

Fig. 1. Map of sampling locations of *Posidonia oceanica* along the Catalan coast and the level of clone sharing between the six patches at each of the three sites: Giverola (a), Cabdells (b) and Fenals (c). We show relative patch size and distance between patches at each location. Numbers at each patch represent the quantity of different genotypes found in each patch. Connecting lines indicate the sharing of clones.



819 **Fig. 2.** Relationship between the response variable 'flower density per patch' and the fixed
820 effects of the best-selected generalised linear model in the 18 *Posidonia oceanica* patches
821 analyzed. (a) Genetic relatedness and (b) heterozygosity had a positive effect on flower
822 density per patch (Table 2). In contrast, the effects of (c) production and (d) genotypic
823 richness were negative (Table 2). Solid lines correspond to the predictions of the best-
824 selected model, shaded areas define the 95% confidence intervals around fitted values and
825 short lines in the bottom of each panel indicate the position of actual observations.



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828 **Fig. 3.** Combined effects of the two most significant predictors of flower density per patch.
829 The highest predicted flower density is for patches with high relatedness and high
830 heterozygosity (red colours), which highlights the additive effects of these two explanatory
831 variables.
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